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(54) Title: IL-6 ACTIVITY INHIBITOR (57) Abstract <p>The invention relates to a nucleotide sequence, which is able to inhibit the IL-6 activity, its use in therapy as well as pharmaceutical compositions containing it. In particular, it relates to a nucleotide sequence which comprises: i) at least one nucleotide sequence that is an APRE element of the general formula ZXMYKGKAA, wherein Z represents T or G or can also be absent, X represents T or can also be absent, M represents C or A, Y represents C or T and K represents T or G, in conjunction with ii) at least one nucleotide sequence constituting a transcription factor binding site other than the APRE element, such as those present in promoter regions.</p>		

IL-6 ACTIVITY INHIBITOR

FIELD OF THE INVENTION

5 The present invention relates to a nucleotide sequence, which is able to inhibit the IL-6 activity, its use in therapy as well as pharmaceutical compositions containing it.

BACKGROUND OF THE INVENTION

10 IL-6 is a protein belonging to the group of cytokines, which proved to play a key role in the organism's immune response and haematopoiesis stimulation.

Many biological functions have, in fact, been found for IL-6 in the hematopoietic and lymphoid system, in the liver and in other target organs and cells. Some of these functions are beneficial, while others are related to pathological states. Among the latter functions, IL-6 has been found to be a growth factor for multiple myeloma cells; anti-IL-6 antibodies were shown to transiently block myeloma cell proliferation in a leukemic patient (see for example Klein et al., Blood, 78, (5), pp.1198-1204, 1991 and Lu et al., Eur. J. Immunol., 22, pp. 2819-24, 1992).

20 Elevated IL-6 levels have been correlated with autoimmune and inflammatory diseases, such as rheumatoid arthritis, glomerulonephritis, psoriasis, and Castelman's disease (see for example Graeve et al., Clin. Investig., 71, pp.664-71, 1993). IL-6 has also been shown to play a direct role in bone loss and hypercalcemia (see for example Poli et al., Embo J., 13, (5) pp. 1189-96 and Yoneda et al., Cancer Res., 53, pp. 737-40, Feb. 1993).

30 The development of inhibitors of IL-6 activity has therefore been the subject of active research. For this purpose, different approaches have been pursued, including the use of antibodies against IL-6 (as reported by Klein et al. above), gp130 or gp80; the use of soluble gp130; or the use of muteins for IL-6, or IL-6 Receptor.

Since these approaches might be associated with specific unwanted effects in clinical applications (as reported by Lu et al., above), the setting-up of additional strategies to inhibit IL-6 activity would be useful.

therefore lead to activation of IL-6-inducible genes (containing such APRE sequences) in IL-6-responsive cells.

As a consequence of this, an APRE element can be used as enhancer of a target gene in IL-6 responsive cells in the following way: in IL-6 responsive cells, the treatment with IL-6 induces the synthesis of APRE proteins, which bind to the APRE element, and such binding activates the expression of the target gene.

Serlupi Crescenzi et al. (Poster at the 12th European Immunol., Meeting, Barcelona, 14-17 June, 1994) have shown that an 8-fold repetition of the APRE DNA sequence (M8) is responsible for a 50-100 fold induction by IL-6 of a reporter gene in HepG2 human hepatoma cells. double-stranded oligonucleotides has in fact been shown for single transcription factors such as .

15 SUMMARY OF THE INVENTION

The main object of the present invention is a nucleotide sequence which is able to inhibit the IL-6 activity, that comprises:

- i) at least one nucleotide sequence that is an APRE element of general formula ZXMYKGKAA, wherein Z represents T or G or can also be absent, X represents T or can also be absent, M represents C or A, Y represents C or T and K represents T or G, in conjunction with
- ii) at least one nucleotide sequence constituting a transcription factor binding site other than the APRE element, such as those present in promoter regions.

Examples of these latter type of nucleotide sequences include: TATA box, and the binding sites for transcription factors, such as AP-1 (see Riabowol et al., PNAS USA, 89, pp. 157-61, 1992), AP-2, HNF-1 (see Clusel et al., Nuc. Ac. Res., 21 (15), pp. 3405-11), SP-1 (see Wu et al., Gene, 89, pp. 203-9, 1990), NF- κ B (see Bielinska et al., Science, 250, pp. 997-1000, 1990), Oct-1, E-2 and SRF transcription factors..

In a preferred embodiment of the present invention, both the APRE element (i) and/or the nucleotide sequence (ii) of the above general formula

are captured by an excess of the nucleotide sequence of the invention, the target gene will not be activated.

According to this assay, inhibitor plasmids are constructed, which contain the nucleotide sequence of the invention. An example of such
5 plasmids is reported in Figure 1 together with its construction strategy.

This and other plasmids, containing the nucleotide sequence of the invention, are also intended to constitute a further embodiment of the invention.

The invention will now be described by means of the following
10 Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction strategy of plasmid pM8SV. pM8SVL is digested
15 with Sal I and Hind III and the sticky ends are transformed in blunt ends by the Kleenow reaction. The resulting 3.2 Kb DNA fragment is purified by agarose gel electrophoresis, then it is self-ligated. Plasmid pM8SV is thus generated, containing the M8 sequence (about 170 bp) and the sequence from the SV40 virus early promoter (about 190 bp), but lacking the
20 luciferase gene.

Figure 2. Sequence of the BamH I-Hind III inhibitor DNA fragment of plasmid pM8SV. The continuous line above the upper part of the shown nucleotide sequence represents the M8 sequence. The bold continuous line above the lower part of the nucleotide sequence represents the SV40
25 promoter sequence. BamH I and Hind III restriction sites are also indicated.

Figure 3. IL-6 reporter gene assay in T47D and M1 cells. Test of different M8-containing plasmids. Reported values of light emission are averages of duplicate determinations of cps integrated over a period of 30 seconds (AUC=Area Under the Curve) from transfected cells. Each bar represents
30 the average of three transfections \pm SEM.

Figure 4. Time-course of luciferase inducibility of transfected Hep G2 cells after treatment with IL-6. Plasmid pM8SVL was used as the positive reporter gene plasmid. Each bar represents the average of two transfections \pm SEM. Two experiments are shown in the Figure.

GGATCCTTCTGGGAATTCTGATCCTTCTGGGAATTCTG (SEQ ID NO: 2). This oligonucleotide was cloned in the Sma I-Bgl II sites of plasmid pGL2-pv (from Promega Corporation), where the expression of the luciferase reporter gene is driven by the SV40 virus early promoter, thus forming, after self-ligation, plasmid pM2SVL.

The synthetic oligonucleotide, through its 5' blunt end, was also ligated to the Sma I site of the same plasmid pGL2-pv and the resulting linear ligation product was then cut with Hind III. The resulting linear vector was used as a recipient to clone the following DNA fragments: 1) the BamH I-Hind III fragment of 238 bp from plasmid pM2SVL, thus forming, after self-ligation, plasmid pM4SVL; 2) the BamH I-Hind III fragment of 280 bp from pM4SVL, thus forming, after self-ligation, plasmid pM6SVL and 3) the BamH I-Hind III fragment of 322 bp from pM6SVL, thus forming, after self-ligation, plasmid pM8SVL.

Plasmid pM8L was constructed by digesting pM8SVL with Sfa I and by converting the sticky ends in blunt ends by fill-in reaction with the Kleenow enzyme. After BamH I digestion, the resulting M8 DNA fragment of 163 bp was ligated with a 16 bp BamH I-Kpn I synthetic adapter and cloned in a 5.6 Kb DNA fragment resulting from Sma I + Kpn I digestion of the pGL2-b vector (from Promega Corporation). Plasmid pGL-2b is identical to the above-mentioned pGL2-pv plasmid, except for the absence of the SV40 promoter sequence in pGL2-b. The 16 bp adapter contained a multiple cloning site, and it was prepared by chemical synthesis with the following sequence:

upper strand: 5'CGCGGCCGCGCTCGAGG3' (SEQ ID NO: 3);
lower strand: 5'GATCCCTCGAGGCGGCCGCGGTAC3' (SEQ ID NO: 4).
The plasmid resulting from the above construction was pM8L, and it had the M8 DNA sequence without promoter, embedded in a multiple cloning site, upstream to the luciferase gene.

The luciferase reporter gene plasmid pM8TKL was prepared by cutting plasmid pGEM-TK-CAT (described in Cohen et al., EMBO J., 7(5), pp. 1411-9, 1988) with Xba I and Bgl II. The resulting 181 bp fragment containing the TK promoter sequence of the viral HSV thymidine kinase

reaction with the Klenow enzyme, purified by agarose gel electrophoresis and then it was self-ligated, thus yielding plasmid pSV which contains the SV40 promoter but lacks the luciferase gene.

The carrier plasmid pC was prepared by cutting plasmid pGL2-b
5 mentioned above with Sal I and Hind III restriction enzymes. The resulting fragment of 2.9 Kb was subject to fill-in reaction with the Klenow enzyme, purified by agarose gel electrophoresis and then it was self-ligated, thus yielding plasmid pC.

All plasmid constructs described above were used to transform the E.
10 Coli strain XL1-Blue with standard techniques (Ausubel R. et al., Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley Interscience, New York). Plasmid DNA was extracted from transformed clones by minipreparative alkaline lysis method (according to Ausubel, above). Plasmid DNA was controlled by restriction analysis and by agarose
15 gel electrophoresis. Clones with the expected pattern were selected. To obtain purified plasmid preparations to be used in transfection of mammalian cells, 300 ml cultures of the selected E. Coli XL1-Blue transformants were prepared. The plasmids were then purified by QIAGEN
tip 500 ion-exchange minicolumns, by following the manufacturer
20 instructions.

Example 2: Cell lines and culture conditions. HepG2 human hepatoma cells (ATCC) were cultured in MEM supplemented with 10% FCS, 5 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin/streptomycin. T47D
25 human breast carcinoma cells (ATCC) were cultured in DMEM supplemented with 10% FCS, 5 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin/streptomycin. M1 mouse myeloid leukaemia cells (ATCC) were cultured in RPMI supplemented with 10% FCS, 5 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin/streptomycin. Culture of the above cell
30 lines was performed in the presence or absence of the appropriate dose of IL-6 (CHO-derived h IL-6 from Interpharm Laboratories), as specified below.

IL-6 was also observed in M1 cells with the reporter gene plasmid pM8TKL, where the M8 molecule was flanked by the thymidine kinase promoter, which is different from the SV40 promoter present in pM8SVL.

Time-course of luciferase inducibility of transfected HepG2 cells has been tested after treatment with IL-6 (1 ng/ml). Plasmid pM8SVL was used as positive reporter gene plasmid (at 0.2 $\mu\text{g}/10^5$ cells). Cells were transfected overnight, splitted and then exposed to the IL-6 treatment. The results are reported in Figure 4 and they show that almost full response to IL-6 could be achieved after only two hours of IL-6 treatment.

10

Example 4: Test of inhibitor plasmid pM8. Inhibition of IL-6 activity by M8 DNA molecules was measured after co-transfection in HepG2 cells of i) the pM8SVL reporter gene plasmid responding to IL-6 and ii) the M8 molecule inserted in the pM8 inhibitor plasmid. This latter plasmid hosts the M8 sequence but it has not the ability to confer responsiveness to IL-6. HepG2 cells were transfected with up to 2.5 $\mu\text{g}/10^5$ cells of the IL-6-responding reporter plasmid pM8SVL (containing M8 and the luciferase gene) and 10 or 50 fold molar excess of pM8, in the presence of various doses of IL-6. The total amount of DNA per transfection was kept constant with the use of the carrier plasmid pC, which is identical to pM8, except for the absence in the former plasmid of the specific 165 bp-long M8 DNA fragment.

As reported in Fig. 5, the inhibitor plasmid did not show significant and reproducible specific inhibition of the IL-6 activity in four experiments, even at 50 fold molar excess of the pM8 inhibitor plasmid. In these experiments the IL-6-responding reporter gene plasmid pM8SVL was used at a dose of 0.1 $\mu\text{g}/10^5$ cells, while the constant amount of total DNA used in transfection was 5 $\mu\text{g}/10^5$ cells. The suboptimal dose of IL-6 used in these experiments was 1 ng/ml. As shown in Fig. 5, variability was acceptable with these experimental conditions, given the fact that distinct transfections per se are an inevitable source of variability.

Example 5: Test of inhibitor plasmid pM8SV. The results reported in Figure 5 were somewhat surprising, because the active M8 inhibitor DNA

same transfection. Reported values of light emission are cps integrated over a period of 30 seconds. As it can be seen from such Table 1, some variability was observed in these experiments, especially at lower doses of the inhibitor plasmid, but usually CVs of replicate transfections were well
5 below 20%.

In order to rule out that the inhibition of IL-6 activity provided by the pM8SV inhibitor plasmid was due exclusively to the SV40 promoter DNA sequence and not to the combination of M8 and SV40 sequences, an additional inhibitor plasmid was constructed and tested in the reporter gene
10 assay. This plasmid contained only the SV40 DNA sequence as inhibitor of IL-6 activity, without the M8 inhibitor sequence, nor the luciferase gene.

Transfections were performed with 0.1 µg of reporter plasmid DNA and the molar excess of inhibitor plasmid shown in Figure 7. The total amount of transfected DNA was kept constant with the carrier plasmid.
15 Transfected cells were treated for 18 hours with 1 ng/ml of IL-6. The results, reported in Figure 7, show that this inhibitor plasmid (pSV) displayed only partial inhibition of IL-6 activity, which never resulted to be above 40% and was not dose-dependent. This allows to conclude that the SV40 DNA sequence alone was not sufficient for effective inhibition of IL-
20 6 activity. On the other hand, the luciferase-containing reporter plasmid pGL2-pv contains the SV40 promoter sequence but not the M8 sequence, thus resulting in a basal level of luciferase expression not further inducible by IL-6. In this plasmid, the basal level of luciferase expression was not inhibited by the pM8SV inhibitor plasmid, thus showing that transcription
25 factors which are specifically bound only to the SV40 promoter region of pGL2-pv, are not effectively removed by the pM8SV inhibitor plasmid. In fact, in the presence of the latter inhibitor plasmid, luciferase activity from the reporter plasmid pGL2-pv was even higher than in the absence of the inhibitor plasmid (not shown).

30

Example 6: Inhibition by pM8SV of the IL-6 activity conferred by the reporter gene plasmid pHPSVL. We then wanted to test the inhibitor plasmid pM8SV in an additional reporter gene assay for IL-6, where the target DNA sequence mediating the IL-6 signal in the reporter gene plasmid

each transfection. 1 and 0.5 μ g of reporter gene plasmid were used in experiments 1 and 2 respectively per 10^5 transfected cells.

Separate transfections of carrier plasmid and inhibitor plasmids were performed in these experiments, both plasmids being used at the indicated
5 molar excess with respect to the reporter plasmid.

After transfection cells were induced for 18 hours with 1 ng/ml of IL-6. Reported values of light emission are cps integrated over a period of 30 seconds. IL-6 specific inhibition in these experiments was evaluated by comparing the induction obtained in the presence of excess of inhibitor
10 plasmid with the induction obtained in the presence of the corresponding dose of carrier plasmid.

Moreover, because of non-specific inhibition, the basal level of luciferase expression was close to the quantitation limit of the assay. Results, reported in Tab. 2 (see Experiment 1) showed that, after triplicate
15 transfections, relevant, specific inhibition of IL-6 activity was obtained at 20-fold molar excess of inhibitor plasmid pM8SV, but not at 10-fold molar excess. A 50-fold molar excess of the inhibitor plasmid could not be tested in this experiment, because non-specific inhibition became too high.

These results could not be reproduced in an additional experiment, when 0.5 μ g DNA / 10^5 cells were used (Tab. 2, see Experiment 2). The
20 reason for this lack of reproducibility can be due to the relatively high variability and low sensitivity of the T47D reporter gene assay. Alternatively, differential target cell selectivity could explain these results.

25 **Example 8: Definition of the minimal DNA inhibitory sequence.** In order to identify the minimal DNA sequence which retains the ability to inhibit the binding of transcription factors relevant for IL-6 inducibility, the experimental approach of electrophoretic mobility shift assay (EMSA) can be used (according to Ausubel). A test for functional inhibition imparted by
30 this minimal sequence can be set using the reporter gene assay mentioned in Example 3 and following Examples. The minimal DNA sequence we have shown to functionally inhibit IL-6 activity in a reporter gene assay was the BamH I-Hind III fragment of 350 bp of the inhibitor plasmid pM8SV, which contains an 8-fold repetition of an APRE DNA sequence and the

Table 1 HepG2 reporter gene assay for IL-6. Test of pSVM8 inhibitor plasmid against pSVM8L reporter gene plasmid

Fold mol. excess of inhibitor	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	- IL-6	+ IL-6	- IL-6	+ IL-6	- IL-6	+ IL-6	- IL-6	+ IL-6
0	30.330	1.562.000	49.200	1.664.000	30.880	2.489.000	37.430	1.587.000
	34.760	1.227.000	83.590	1.914.000	47.610	2.266.000	34.000	1.433.000
	32.920	1.711.000	91.560	1.754.000	49.670	3.239.000	31.130	1.362.000
10	29.400	751.800	144.300	2.380.000	33.680	923.100	32.390	476.200
	34.660	951.300	161.500	2.202.000	35.020	722.500	39.610	790.400
	24.530	1.159.000	129.200	1.953.000	37.620	1.110.000	32.770	516.900
25	40.730	568.100	85.410	745.300	46.760	538.400	36.790	298.100
	45.320	677.700	61.340	671.600	33.520	414.000	20.320	255.500
	32.670	620.400	71.040	773.300	27.950	543.500	26.080	318.800
50	53.210	312.200	148.700	857.900	23.820	173.500	120.800	586.000
	60.110	268.500	159.700	655.300	25.370	150.500	96.900	716.400
	73.810	427.300	68.630	517.500	46.470	287.500	195.800	1.029.000

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.
- (B) STREET: 6 JOHN GORSIRAWEG
- (C) CITY: CURACAO
- (E) COUNTRY: NETHERLANDS ANTILLES
- (F) POSTAL CODE (ZIP): NONE

(ii) TITLE OF INVENTION: IL-6 ACTIVITY INHIBITOR

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 356 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCCAGGATCC TTCTGGGAAT TCTGATCCTT CTGGGAATTC TGATCCTTCT GGAATTCTG 60
ATCCTTCTGG GAATTCTGAT CCTTCTGGGA ATTCTGATCC TTCTGGGAAT TCTGATCCTT 120
CTGGGAATTC TGATCCTTCT GGAATTCTG ATCTGCATCT CAATTAGTCA GCAACCATAG 180
TCCCGCCCCT AACTCCGCCC ATCCCGCCCC TAACTCCGCC CAGTTCCGCC CATTCTCCGC 240
CCCATGGCTG ACTAATTTTT TTTATTTATG CAGAGGCCGA GGCCGCCTCG GCCTCTGAGC 300
TATTCCAGAA GTAGTGAGGA GGCTTTTTTTG GAGGCCTAGG CTTTTCGAAA AAGCTT 356

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GATCCCTCGA GCGGCCGCG GTAC

24

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTACGCGTGC AGTATTGACC CTCCTCCT

29

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGCAGATCTA GCTCACTTCT CCCCCTTC

28

8. The nucleotide sequence according to any preceding claim, wherein the sequence (ii) comprises at least two different oligonucleotide sequences constituting a transcription factor binding site.
- 5 9. The nucleotide sequence according to claim 8, wherein the sequence (ii) is the SV40 early promoter.
- 10 10. The nucleotide sequence according to claim 1, as reported in SEQ ID NO: 1.
11. A plasmid vector containing the nucleotide sequence of any preceding claim.
- 15 12. Use of the nucleotide sequence according to any of claims from 1 to 10 in therapy to inhibit the action of IL-6.
- 20 13. A pharmaceutical composition comprising the nucleotide sequence according to any of claims from 1 to 10 together with one or more pharmaceutically acceptable carriers and/or excipient.
14. A pharmaceutical composition comprising the plasmid according to claim 11 together with one or more pharmaceutically acceptable carriers and/or excipients.

Figure 2 **Sequence of the BamH I-Hind III inhibitor DNA
fragment of plasmid M8SV**

```
BamHI \ _____  
CCCAGGATCC TTCTGGGAAT TCTGATCCTT CTGGGAATTC 40  
_____  
TGATCCTTCT GGGAATTCTG ATCCTTCTGG GAATTCTGAT 80  
____ M8 sequence _____  
CCTTCTGGGA ATTCTGATCC TTCTGGGAAT TCTGATCCTT 120  
_____/ \ _____  
CTGGGAATTC TGATCCTTCT GGGAATTCTG ATCTGCATCT 160  
_____  
CAATTAGTCA GCAACCATAG TCCCGCCCCT AACTCCGCCC 200  
_____  
ATCCCGCCCC TAACTCCGCC CAGTTCCGCC CATTCTCCGC 240  
_____ SV40 sequence _____  
CCCATGGCTG ACTAATTTTT TTTATTTATG CAGAGGCCGA 280  
_____  
GGCCGCCTCG GCCTCTGAGC TATTCCAGAA GTAGTGAGGA 320  
_____/ _____  
GGCTTTTTTG GAGGCCTAGG CTTTTCGAAA AAGCTT 356  
Hind III
```

Figure 4 Time course of luciferase inducibility by IL-6 in HepG2 cells

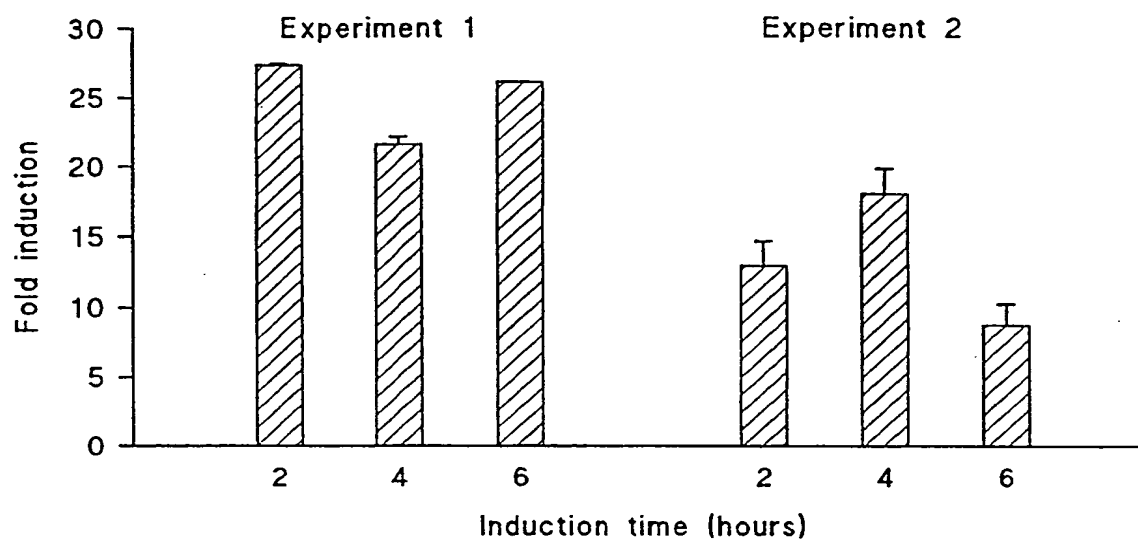


Figure 6 **HepG2 reporter gene assay for IL-6. Test of pM8SV inhibitor plasmid against pM8SVL reporter gene plasmid**

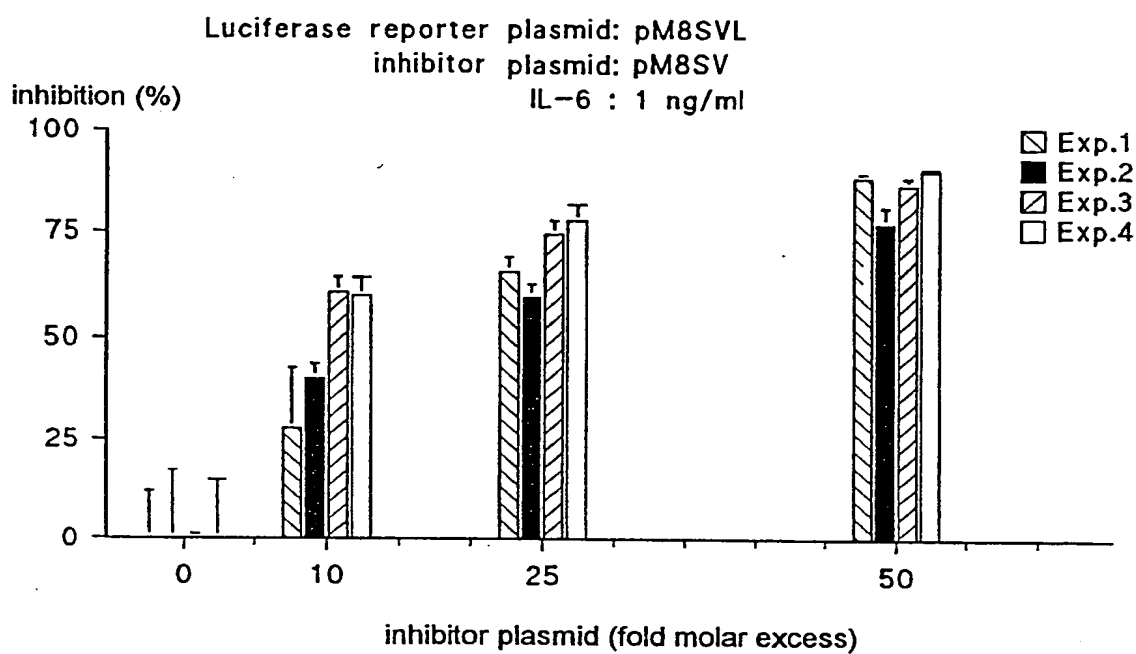
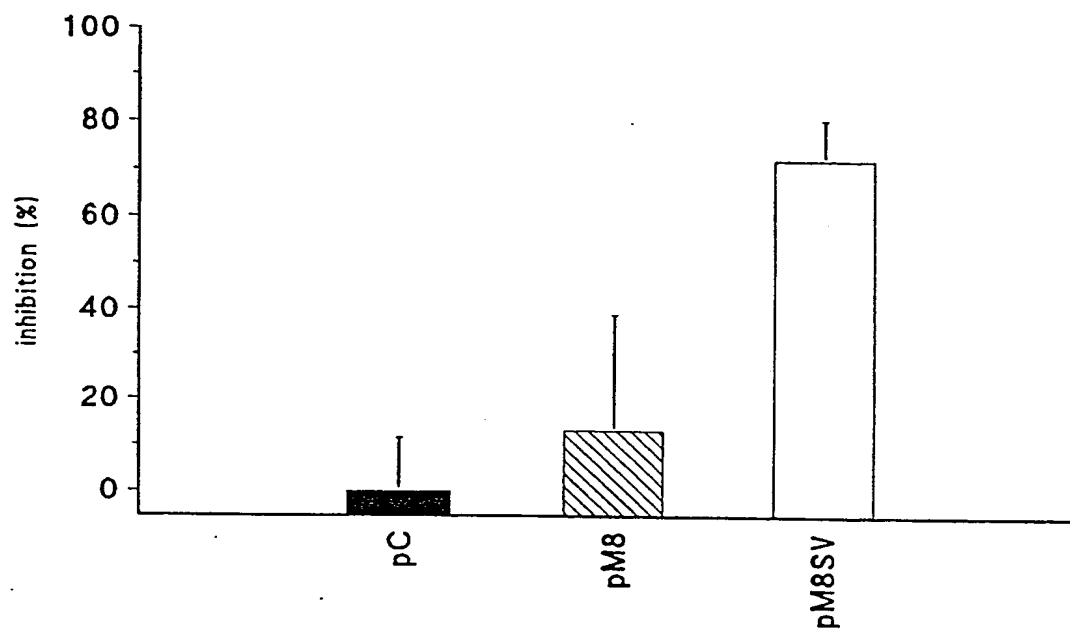


Figure 8 **HepG2 reporter gene assay for IL-6. Test of inhibitor plasmids against pHPSVL reporter gene plasmid**

Luciferase reporter plasmid: pHPSVL
inhibitor plasmids: as shown, at 50 fold molar excess
IL-6: 1 ng/ml



INTERNATIONAL SEARCH REPORT

Int. Appl. No.
PCT/EP 95/01778

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *

Citation of document, with indication, where appropriate, of the relevant passages

Relevant to claim No.

Y

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vol. 14, no. 3, 1994
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factors which bind to common responsive
DNA elements'
see the whole article

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oligodeoxyribonucleotides'
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oligonucleotides'
see the whole article

1-14